Magnesium *vs.* manganese cofactors for metallonuclease enzymes. A critical evaluation of thermodynamic binding parameters and stoichiometry

Ruby Leah B. Casareno and J. A. Cowan*

Evans Laboratory of Chemistry, The Ohio State University, 100 West 18th Avenue, Columbus, OH 43210, USA

An experimental analysis of Mg^{2+} *vs.* Mn^{2+} binding to *Escherichia coli* ribonuclease H and exonuclease III enzymes by isothermal titration calorimetry clearly demonstrates a 1:1 stoichiometry for metal binding to ribonuclease H, but distinct metal-dependent behaviour for exonuclease III, suggesting caution in the interpretation and generalization of results concerning the location and stoichiometry of Mg^{2+} binding sites from crystallographic and mechanistic experiments with Mn^{2+}.

The mechanism of metal-ion-mediated phosphate ester hydrolysis by enzymes and ribozymes is an important issue in nucleic acid biochemistry.[1,2] The stoichiometry of metal cofactor required to effect hydrolysis has been the subject of speculation,[3–7] with much of the current thinking on this subject being inferred from analysis of a rather small body of crystallographic data,[4,5] with relatively few results from solution experiments.[3] In spite of the '*common awareness*' that the metallobiochemistry of transition-metal ions (typically Mn^{2+} or Co^{2+}) may differ from Mg^{2+},[8] these analogue cofactors are commonly used without a critical appraisal of the significance of the results. In this paper, we have carried out a comparative study of the binding of Mg^{2+}, Ca^{2+} and Mn^{2+} to *E. coli* ribonuclease H (RNase H) and exonuclease III (exo III) enzymes. These experiments characterize the thermodynamics of metal ion binding and selectivity, and clearly show a 1:1 stoichiometry for metal binding to RNase H for Mg^{2+}, Ca^{2+} and Mn^{2+}. However, exonuclease III binds at least two Mn^{2+} ions, in sharp contrast to results obtained for Mg^{2+} and Ca^{2+} which show a single site. Inasmuch as crystallographic and mechanistic studies of magnesium-dependent nucleases often substitute Mn^{2+} for Mg^{2+}, these results carry clear implications for the interpretation of such experiments, and for the important issue of *one-metal-ion vs. two-metal-ion* mechanisms for nucleic acid hydrolysis.

Metal ion binding to *E. coli* ribonuclease H and exonuclease III was evaluated by isothermal titration calorimetry.[9] Table 1 summarizes the thermodynamic parameters obtained for metal binding to RNase H. In each case the fit yielded one metal ion binding site. The binding entropy is positive for both Mg^{2+} and Mn^{2+} [9.7 and 5.7 cal K^{-1} mol^{-1}, respectively (cal = 4.184 J)], consistent with the release of several water molecules as suggested by crystallographic data. The carboxylates of Asp10 and Glu48, and the backbone carbonyl of Gly11 have been shown to coordinate to Mg^{2+}.[6] In contrast, the binding entropy for Ca^{2+} is negative (−10.4 cal K^{-1} mol^{-1}). Most likely the larger Ca^{2+} ion can coordinate to the catalytic base Asp70.[10] Subsequent ordering of residues in the active site can presumably compensate for the release of up to four water molecules displaced from hydrated Ca^{2+}, and would explain both the negative binding entropy and the negligible level of activity observed for the Ca^{2+}-bound enzyme.[11] The Mn^{2+} binding stoichiometry that we have obtained for *E. coli* RNase H contrasts with crystallographic data obtained with the structurally homologous RNase H domain of HIV reverse transcriptase, which shows two Mn^{2+} ions located among four acidic residues (Asp443, Glu478, Asp498, and Asp549) in a Mn^{2+}-doped crystal.[5] These acidic residues are four of the seven conserved residues found in all bacterial and retroviral RNase H domains, including the *E. coli* enzyme. Either the HIV-RT RNase H domain does indeed bind two divalent metal ions, or this result reflects the specific use of Mn^{2+} and/or the doping technique employed.

The issue of metal cofactor stoichiometry is important and of general relevance to the understanding of metal-mediated nucleic acid hydrolysis. The possibility of distinct metal binding stoichiometries, according to the selection of cofactor, has not yet been clearly demonstrated for a metallonuclease enzyme. We now report one such example. Table 2 summarizes calorimetry plots and parameters for metal binding to exo III. The structure of this DNA repair enzyme has recently been established.[12] In contrast to RNase H, there appears to be only one metal-coordinating ligand (Glu34) in the active site. The binding parameters for Mg^{2+} and Ca^{2+} are similar, with positive entropy terms that are consistent with the release of a water

Table 1 Thermodynamic parameters for metal ion binding to *E. coli* ribonuclease H[a]

Cofactor	10^{-4} K/ dm^3 mol^{-1}	ΔH/ kcal mol^{-1}	ΔG/ kcal mol^{-1}	ΔS/ cal mol^{-1} K^{-1}
Mg^{2+}	1.1 ± 0.2	−2.6 ± 0.1	−5.5 ± 0.1	9.7 ± 0.7
Ca^{2+}	44 ± 12	−10.8 ± 0.2	−7.7 ± 0.1	−10.4 ± 0.6
Mn^{2+}	5.9 ± 0.6	−4.8 ± 0.2	−6.5 ± 0.1	5.7 ± 0.8

[a] Data from the calorimetric titration of RNase H (0.17 mmol dm^{-3}) with 20 × 12.5 μl injections of metal ion (2 to 10 mmol dm^{-3}) at 298 K in 20 mmol dm^{-3} Tris, pH 7.0, 1 mmol dm^{-3} HSC_2H_4OH, obtained from the average of at least two independent experiments following published instrumental procedures.[9] Experiments were carried out with appropriate controls, correcting for heats of dilution when necessary.

Table 2 Thermodynamic parameters for metal ion binding to *E. coli* exonuclease III[a]

Cofactor	10^4 *K*/ dm^3 mol^{-1}	ΔH/ kcal mol^{-1}	ΔG/ kcal mol^{-1}	ΔS/ cal mol^{-1} K^{-1}
Mg^{2+}	0.91 ± 0.02	−4.6 ± 0.1	−5.4 ± 0.3	2.7 ± 0.3
Ca^{2+}	1.8 ± 0.3	−5.3 ± 0.2	−5.8 ± 0.1	1.7 ± 0.2
Mn^{2+} (type 1)[b]	16 ± 2	−8.9 ± 0.2	−7.1 ± 0.3	−6.0 ± 0.8
Mn^{2+} (type 2)[b]	2.5 ± 0.3	1.0 ± 0.1	−6.0 ± 0.3	16.8 ± 0.9

[a] Data from the calorimetric titration of exo III (0.17 mmol dm^{-3}) with 16 × 12.5 μl injections of metal ion (10 mmol dm^{-3}) at 298 K in 20 mmol dm^{-3} Tris, pH 7.5, 1 mmol dm^{-3} HSC_2H_4OH. Other procedures are described in the legend to Table 1. [b] These parameters are from one of several possible fits to the data. At least two classes of site are required; one displaying exothermicity and the other endothermicity in Mn^{2+} binding.

ligand. For both of these ions one binding site was available. In contrast, the binding profile for Mn^{2+} was distinct with clear evidence for (at least) two classes of site, one showing exothermic binding and the other showing endothermic binding. The X-ray structure of Tainer and coworkers shows only one bound Mn^{2+}; presumably since the weaker site(s) is not populated under the crystallization conditions employed.[12] The distinct peaks observed at each injection suggest that binding to the endothermic sites is considerably faster than binding to the exothermic sites. The number of ions at each type of site can vary, resulting in distinct binding parameters; however, a *combination* of endothermic and exothermic sites are always obtained, and clearly demonstrate the distinct binding behaviour of Mn^{2+} relative to Mg^{2+}. Since divalent manganese is commonly used as a probe for Mg^{2+} chemistry in solution and crystallographic studies, our data suggest a note of caution against the generalization and interpretation of results concerning the location and stoichiometry of metal binding from such experiments. This conclusion is particularly pertinent to the issue of *one-metal-vs. two-metal-ion* mechanisms in phosphate ester hydrolysis.

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